Induced Engulfment of *Neisseria gonorrhoeae* by Tissue Culture Cells

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Engulfment of gonococci by mammalian tissue culture cells was examined as a model of the penetration of host cells in gonorrhea. Engulfment required viable organisms; killing the gonococci with heat or refrigeration abolished the process. Engulfment also required tissue culture cell microtubule- and microfilament-dependent movement; treating the cells with cytochalasin B (0.5 μg/ml) or demecolcine (Colcemid; Ciba-Geigy AG, Basel, Switzerland) (10 μg/ml) also prevented this process.

The presence of intraepithelial cell gonococci (GC) was noted 40 years ago (3), and it has been speculated that this intracellular existence is both a path to tissue invasion and a mechanism of protection of the bacteria from host immune mechanisms (6). The presence of intracellular organisms has also been demonstrated in vitro in organ cultures (2) and tissue cultures (9), and electron micrographs have been interpreted as showing active engulfment of GC on the part of host epithelial cells (10). This work furthered these observations by investigating whether the engulfment is sufficiently complete to allow protection of GC from the bactericidal effects of specific antibody and complement, what active role is played on the part of the host cells, and whether the reaction is triggered by a stable constituent of GC which would lend itself to fractionation and analysis or requires viable organisms to proceed.

GC isolates (from a local venereal disease clinic) which had been passed no more than four times were divided into aliquots, frozen, and stored at −70°C until needed. Type 1, virulent, opaque colonies were used in all experiments. Hyperimmune rabbit immunoglobulin G (anti-GC) conjugated to horseradish peroxidase (HRP) was tested against the GC grown on solid media and in liquid tissue culture (TC) media, and only those conjugates and GC strains which yielded strong staining of all organisms were used. Three different isolates were used in these experiments, with equivalent results. When needed, organisms were thawed, plated on enriched chocolate agar (GC medium base [Difco Laboratories, Detroit, Mich.], 1% hemoglobin [Difco], 1% IsoVitalex [BBL Microbiology Systems, Cockeysville, Md.]) at a density of 10⁶ organisms per plate, incubated in 10% CO₂ at 37°C for 14 to 16 h, and then suspended directly in the TC media for inoculation of TCs.

Baby hamster kidney cells (BHK-21) and human epithelial cells (HeLa) (both from M.A. Bioproducts, Walkersville, Md.) and human amnionic cells (HAC) from primary cultures were maintained in medium 199 (Difco) plus fresh glutamine and 5% fetal calf serum. Cells for inoculation with GC were grown to confluence, inoculated with the suspension of GC, and incubated at 37°C in 5% CO₂ for 6 h. The inoculation medium was then removed, medium containing HRP-anti-GC was added, and incubation was continued for 5 min. The TC monolayer was washed three times with Hanks balanced salt solution, fixed with neutral buffered 4% Formalin, reacted with 0.1% hydrogen peroxide and diamino-benzidine (50 mg/dl), and then counterstained with Giemsa stain. Extracellular bacteria were stained brown by the HRP-anti-GC-diamino-benzidine combination. Intracellular bacteria failed to bind the conjugate and were stained blue by the counterstain. The relative positions of diamino-benzidine-positive and -negative GC were confirmed by transmission electron microscopy (Fig. 1). These preparations were processed as described above except that after reacting with the diamino-benzidine they were fixed with 1% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded by conventional techniques.

After 6 h of incubation of GC with TC cells, intracellular GC were readily detected in BHK-21 cells (means, 0.87 ± 0.18 intracellular GC and 3.0 ± 4.1 extracellular GC per TC cell for 300 TC cells counted). Trials with HAC cells produced equivalent results, while very few intracellular GC were detected in HeLa cells. BHK-21 cells were used in all subsequent experiments. Five percent fetal calf serum was routinely used in the GC inoculation medium because it was found that when this serum was eliminated there was little or no engulfment.

The mechanisms involved in GC engulfment were then investigated by blocking microtubular action with demecolcine (Colcemid; Ciba-Geigy AG, Basel, Switzerland) and microfilament action with cytochalasin B. At 5 min before inoculation of BHK-21 cells with GC, the standard medium was exchanged for the same medium containing cytochalasin B or demecolcine. The GC inoculum was also prepared with the same concentrations of these drugs just before being added to TC cells. When BHK-21 cells were treated with demecolcine at 4 μg/ml or cytochalasin B at 0.1 μg/ml, engulfment was partially suppressed, while treatment with demecolcine at 6 to 10 μg/ml or cytochalasin B at 0.5 to 1.0 μg/ml completely suppressed GC engulfment. GC exposed to demecolcine or cytochalasin B under the above-described conditions grew normally when replated on agar.

The requirements of active bacterial participation in engulfment were tested by heating or cooling the GC prior to adding them to TC cells. Heating the GC to 56°C for 30 min or cooling them to 4°C for 18 h, after which no viable bacteria could be detected, completely prevented engulfment of these GC by BHK-21 cells.

Finally, the viability of engulfed GC was tested by exposing infected TC cells to antisera and complement. The
medium of the inoculated TCs was replaced with medium containing 5% rabbit anti-GC antiserum and 10% fresh rabbit serum and incubated for 1 h at 37°C. The TC cells were then rinsed three times with Hanks balanced salt solution and trypsinized to completely disperse them. Log dilutions were made in Hanks balanced salt solution, and aliquots were plated on enriched chocolate agar. The agar plates were incubated, and GC CFU were counted by standard techniques. CFU per milliliter of trypsinized BHK-21 cells, at four trials for each point, were $22 \times 10^6$ (range, $0.8 \times 10^6$ to $45 \times 10^6$) with no antiserum $9.4 \times 10^6$ (range, $1.4 \times 10^6$ to $28 \times 10^6$) when rabbit anti-GC antiserum and complement were added, $7.0$ (range, $0.2 \times 10^6$ to $15 \times 10^6$) when cytochalasin B was used without antiserum, and $36$ (range, $0$ to $200$) when antiserum and cytochalasin B were used. Thus, engulfment of GC by BHK-21 cells provided protection from the bactericidal effects of the anti-GC antibodies and complement, while few or no GC survived when engulfment was blocked with cytochalasin B. Neither cytochalasin B by itself, antisem by itself without fresh complement, nor the source of complement without antiserum had any significant effect on the viability of GC.

Demecolcine and cytochalasin B are drugs which block the actions of the microtubule and microfilament systems, respectively, of mammalian cells (4, 5), and cytochalasin B has recently been used to block the uptake of GC by TC cells (1, 8). These and the present observations suggest that both microfilaments, which are associated with chemotaxis and pseudopod formation, and microtubules, which are associated with phagocytosis and cell movement (6, 7), are needed for GC engulfment.

In addition, engulfment appears to require viable GC. Organisms heat killed or killed by refrigeration overnight were not engulfed, suggesting that a labile bacterial factor triggers the engulfment. Engulfment was also dependent on the presence of serum in the TC media, suggesting that a serum factor may act as an intermediary in this reaction. Once engulfed, the bacteria proved to be resistant to the bactericidal actions of immune serum and complement, in agreement with the results of Bessen and Gotschlich (1).

Most of the well-known gonococcal antigens, such as pili or capsular polysaccharide, which have been investigated to date for use as a vaccine have shown too much variation from one isolate to another to be of use in conferring broad protection (7). Host epithelial cell engulfment has been recognized as part of the pathogenesis of gonorrhea for many years, but too little has been known about the specific substances involved to use them in any approach to disease prevention. The present results suggest that there is a substance produced by GC which triggers engulfment by host epithelial cells and that this substance is either quite labile or quickly lost from nonviable organisms. Because the engulfment reaction is not restricted to a specific TC cell type or GC isolate, the receptor and its ligand may be relatively constant. If this is true, this substance may lend itself to the production of a broadly protective vaccine. However, initial attempts to identify such a substance in the filtrate of viable organisms have met with equivocal results.

LITERATURE CITED


